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CO_2 leakage simulation: Effects of the decreasing pH to the survival and reproduction of two crustacean species

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ABSTRACT

The effects of CO_2-related acidification on two crustacean populations, the isopod *Cyathura carinata* and the amphipod *Elasmopus rapax*, were studied. Three pH levels were tested: artificial seawater without CO_2 injection and two levels of reduced pH. Even though RNA:DNA ratio was reduced for both species, no statistical significant differences were found between the control and the treatments. Both species experienced a reduction in survivorship, longevity and the body length of surviving animals; although the impairment observed in *E. rapax* was more severe than in *C. carinata*. The long life span isopod and the short life span amphipod experienced a high degree of impairment in the reproduction, likely due to the reallocation of resources from reproduction to body maintenance and increasing survival by postponing the brood production. Regardless of the underlying processes and the energetic pathways, both experienced failure to reproduce, which could lead to the local extinction of these species.

1. Introduction

Global climate change (GCC) is considered an indisputable reality (IPCC, 2005) that presents an unprecedented threat to global marine biodiversity. The oceans act as a buffer against climate change by absorbing CO_2 from the atmosphere; this results in a reduction in ocean pH levels that has adverse impacts on the numerous ecosystem services that we obtain from the oceans (Barry et al., 2011). Carbon dioxide capture and storage (CCS) technology is considered to be a promising option in the portfolio of mitigation measures against GCC (IPCC, 2005; Reguera et al., 2009). The "E.U. 2030" climate and energy policy framework refers to the important role that CCS could play for the long-term reduction of emissions from industrial processes and for the stabilisation of atmospheric greenhouse gas concentrations. To date, many countries around the world are interested in CCS in stable geological formation projects (Global CCS Institute, 2018). Most of those projects are in the operational phase, such as Sleipner West and Snøhvit (Norway), Weyburn CO_2 Flood Project (Canada), In Salah (Algeria), K12B (Holland) and La Barge (Wyoming), with the capacity to capture up to 28 million tons of CO_2 per annum (MTPA) (Global CCS Institute, 2015). Additionally, 20 other projects are currently in the execution or the advance planning stages, which will greatly increase the MTPA of CO_2 captured during the next few years. Despite the worldwide interest in the application of CCS techniques, there are hardly any specific standards for its implementation, with more attention focused on the CO_2 capture and transport procedures than on gas injection and storage. Furthermore, there is a lack of awareness by governments and international institutions about the environmental implications of CSS technology, so the development of a suitable international framework for onshore and offshore CO_2 storage is still needed. Despite the fact that this technology is well established, the reasonable cost and the possible success in reducing CO_2 concentrations, there are some risks associated with the process of capture and storage (Bouc et al., 2009; Damen et al., 2006; Gale, 2004). In particular, there is a potential for CO_2 leakage from the capture facilities, transport pipelines and offshore installations, which could have negative consequences on the environment. Understanding of CO_2 behavior in case of a leak is currently low, as the underground storage of CO_2 is still a relatively young technology (Damen et al., 2006). The Global Status of CCS Report (Global CCS Institute, 2018) inventories 43 large-scale facilities (18 in

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commercial operation, five under construction and 20 in various stages of operation). However, the difficulty of predicting the location and magnitude of seepages complicates the evaluation of potential effects on the ecosystem.

Recently, shallow in situ injection experiments have been used to simulate controlled CO_2 leaks in an effort to observe the behaviour of CO_2 plumes under release events (Blackford et al., 2013, 2014; Blackford and Kita, 2013; Denchik et al., 2014; Jones et al., 2014). Roberts and Stalker (2017) reported release of 82.8 t CO_2 over 992 days, and concluded that cost-effective approached for monitoring includes remote sensing methods or shallow CO_2 release experiments.

CO_2-related acidification scenarios (ocean acidification, CCS leaks, natural sources, etc.) could cause serious environmental perturbations to marine ecosystems because it is known that sudden changes in pH provoke acute adverse effects on marine biota (i.e., Basallote et al., 2012, 2014, 2015, 2017; Calosi et al., 2013; De Orte et al., 2014b; Díaz-García et al., 2017; Payán et al., 2012; Rodríguez-Romero et al., 2014, 2016). Lethal effects on marine organisms could appear if the pH falls below a level that can be tolerated by a specific organism, although the lowest pH or the lethal CO_2 concentrations would largely vary between species, life stages, and even within taxonomic groups (i.e., Byrne, 2011; Kurihara and Ishimatsu, 2008; Ries et al., 2009). Furthermore, local adaptation and variability between individuals or populations, and physiologic plasticity of the communities being exposed to these conditions will also influence the toxic effects of the CO_2-related acidification (Lee et al., 2016). For these reasons, it is recommend the inclusion of biological observations for monitoring and for monitoring and assessing relevant impacts under scenarios of rapid ocean acidification (e.g., Blackford et al., 2014). Other authors have also stated that the environmental effects of possible CO_2 escapes is an important issue to be included in CCS technology risk assessment studies (Carrol et al., 2014; DeVries et al., 2013). Lessin et al. (2016) have also suggested that efficient monitoring and leak mitigation strategies, coupled with appropriate selection of storage sites, can effectively limit concerns regarding localised environmental impacts caused by CCS.

The present paper addresses the potential effects of rapid CO_2-related acidification event and its possible consequences on two crustacean species from marine environments (the isopod *Cyathura carinata* and the amphipod *Elasmopus rapax*). *C. carinata* and *E. rapax* are representative species of marine habitats with wide geographical distribution but with very different length in their respective life cycles (Ferreira et al., 2004; Hughes and Lowry, 2010), so this work can also compare whether rapid CO_2-related acidification affects differently to two crustaceans species with different modes of lifespan. A second goal of this research was to make use of sensitive molecular techniques, such as RNA:DNA ratio, to identify sub-lethal effects that might be undetected in individual performance in response to OA (e.g. individual mortality or growth rate).

2. Materials and methods

2.1. Animal and sediment collection and pre-experimental conditions

The isopod *Cyathura carinata* (Krøyer, 1847) is a key species of estuarine habitats (Bordalo et al., 2011) with a wide geographical distribution (the Atlantic coasts of Europe, Baltic and Mediterranean seas) with a relatively long life span (i.e. up to 2 years) (Ferreira et al., 2004). Stocks of *C. carinata* were collected from the Odiel marshes, which is a nature landscape and Reserve of the Biosphere established by the UNESCO (37°08′N to 37°20′N, 6°45′W to 7°02′W; Southwestern Iberian Peninsula) far away of the industrial area (pH 7.8–8.0 according to the General Directorate of Prevention and Environmental Quality Laboratory, Regional Ministry of the Environment of Andalucía). The isopods were collected by sieving mud through a 1 mm mesh in the intertidal zone (upper 5 cm).

The marine amphipod *Elasmopus rapax* (Costa, 1853) used in toxicological studies (Bao et al., 2012; Zanders and Rojas, 1992) was selected due to its short life span (30-days; Hughes and Lowry, 2010). Hughes and Lowry (2010) indicate that this amphipod lives in a very stable marine habitat with a rather constant pH and have an extensive geographical distribution through both tropical and temperate regions. Amphipods were collected from the port of a recreational boating marina, the Puerto América Cádiz Marina, located on the Atlantic coast of Southern Spain (36°32′29″ N, 6°17′61″W). This area is characterized by a quasi constant pH (pH 7.8–8.0) and oxygen concentrations through the year with no statistical differences among them (Ros et al., 2013). The specimens were collected together with the substratum (e.g. bryozoans) on which they lived.

After sampling, both species were kept in aerated and filtered clean artificial seawater (Tropic Marin© SEA SALT) with a salinity of 34 ± 1 psu, temperature of 20 ± 1 °C and a pH_{NBS} equal to 7.98, resembling conditions at the sampling area during their collection, and they were acclimated to 12 h:12 h photoperiod and oxygen levels > 80% during the two weeks prior to the experiment. During this period, *C. carinata* and *E. rapax* were fed once and twice per week with Gamma Nutraplus (Tropical Marine Centre) ad libitum, respectively. Furthermore, *C. carinata* were fed once per week with polychaetes due to wide diet and aggressive behaviour (Ferreira et al., 2004). Both species of crustaceans were classified according to their size to select a homogeneous class size for the test.

Since water acidification affects the stability of metal(loid)s trapped on marine sediments (De Orte et al., 2014a; Payán et al., 2012; Roberts et al., 2013; Rodríguez-Romero et al., 2014) and the Odiel marshes have a high and variable metal concentration in their sediments (Blasco et al., 2010; Márquez-García et al., 2013), the sediment used for the incubations was collected from the surface sediment (5 cm) of an unpolluted area (Espigón Beach −37°08′N, 6°53′42″W). Sediment was sieved to remove associated macro-fauna and large debris (0.5 mm mesh size), and placed in pre-cleaned polypropylene containers. The buckets were filled to the brim and hermetically closed. Sediment samples were transported to the laboratory where they were kept at 4 °C in darkness prior to the experiment (no longer than 10 days).

2.2. Experimental setup and physico-chemical parameters

The CO_2 system, with a non-pressurised chamber and a CO_2 injection into both the water (*E. rapax*) or into the sediment (*C. carinata*) according to the life-style of each organism, has been previously described by Basallote et al. (2014), De Orte et al. (2014a), and Rodríguez-Romero et al. (2014). Aqua Medic AT Control System (Europe) was used to control and maintain the pH in each tank where the pH electrodes were placed. Before use, pH electrodes of the CO_2-injection system were calibrated (SD ≤ 0.03) and the values obtained throughout the tests were regularly verified by a portable pH-meter (Phenomenal 1000H; accuracy ± 0.005 pH). A solenoid valve allowed adjustment of pH values when it was detected that the pH had increased by 0.01 units or more; then, CO_2 gas bubbles were injected into each tank until the required pH value was reached. A computer connected to the AT control system allowed to modify the pH values, as required. In this experiment, all exposure tests were carried out in a temperature- and light-controlled chamber (20 ± 1 °C and a photoperiod of 12:12 h light: dark).

The pH exposure levels were selected based on predicted pH reduction scenarios caused by CO_2 leakages in marine ecosystems and the expected pH-related mortality for these species (Basallote et al., 2014). All electrodes were calibrated before use. In order to verify the pH values during the experiment, results for normality (Kolomogorov–Smirnov test), and then we carried out a one-way analysis of variance (ANOVA) for the *C. carinata* experiment and a Kruskal–Wallis test for the *E. rapax* experiment.

Total alkalinity (TA) values were determined from 50 mL seawater aliquots using an automatic titrator (Hanna instruments HI 84431) with

a glass-combined electrode. The TA results were expressed as μmol/kg. Carbon system speciation was calculated using the CO2SYS programme (Pierrot et al., 2006), with a dissociation constant as described by Mehrbach et al. (1973), with refit as described by Dickson and Millero (1987) and $KHSO_4$ as described by Dickson (1990).

2.3. Elasmopus rapax experiment

Amphipods were randomly placed in groups of 11 animals (average size = 5.02 ± 0.32 mm) in each aquarium of the CO_2 injection system, with at least two males per aquarium. The animals were maintained in acid-washed 5 L glass tanks containing 2 L of continuously aerated seawater. Three pH levels were tested, with three replicates for each treatment: artificial seawater without CO_2 injection (control: nominal pH = 8.0) and two levels of reduced pH (nominal pH = 7.5 and 7.0). Seawater pH was reduced gradually (0.5 units/day) until the experimental pH value was reached (Basallote et al., 2014; Rodríguez-Romero et al., 2014), then, the assay was started. Each aquarium was covered by a breathable sealing film that allowed gas exchanges, whilst reducing evaporation and, thus, avoiding salinity and temperature fluctuations. The conditions of the cultures were the same as *E. rapax* stock (see Table 1). Surviving animals were counted once per week, and the maturity of each individual was noted. At the end of the experiment (22 days), amphipods were measured to the nearest 0.05 mm.

2.4. Cyathura carinata experiment

Isopods were randomly placed in groups of 20 animals (average size = 9.5 ± 2.5 mm) in each aquarium with the CO_2 injection system. At least two males were included per aquarium (large specimens lacking oostegites). The animals were maintained in acid-washed 25 L glass tanks containing 10 L of continuously aerated seawater overlying 5 cm of sediment (sieved by 0.05 mm). After the sediment settled to the bottom of the tank, the isopods were introduced and exposed for 32 days to one of three pH levels: artificial seawater without CO_2 injection (control: nominal pH = 8.0) and two levels of reduced pH (nominal pH = 7.0 and 6.5). The acclimatising process was similar to the *E. rapax* experiment. The conditions of the cultures were similar to the *C. carinata* stock population (see Table 1). The sediment was sieved once per week; the surviving animals were counted and their development stage annotated. At the end of experiment, animals were measured to the nearest 0.05 mm.

2.5. Endpoints

2.5.1. RNA:DNA ratio

E. rapax individuals were collected for biochemical analysis after 8 days of exposure to a pH of 7.0 and after 22 days of exposure in the control and pH 7.5 scenarios. *C. carinata* individuals were collected for biochemical analysis after 24 h, and after 7, 28 and 32 days of exposure. These different sampling periods are related with the resistant of acidification in both species. Each crustacean species was cleaned, as described by Bao et al. (2012), placed into a previously weighted nuclease-free Eppendorf tube, weighted in a precision balance and immediately frozen at −80 °C. Methods for quantification of nucleic acids largely followed the method described in Vrede et al. (2002), with some modifications. RNA and DNA were extracted and analysed using the fluorochrome RiboGreen in combination with RNase treatment (Gorokhova and Kyle, 2002). Briefly, Extraction buffer (0.1% Triton X-100) and protease (0.1 mg/mL final concentration) was added to each Eppendorf vial, crushed with a homogenizer (Micra, D-1 model) and shaked during 1 h. The crude homogenates were centrifuged at 2000*g* for 1 min and fluorescence measurements were performed (BioTek Synergy HT). Samples were prepared in duplicate. Later on, RiboGreen (Molecular Probes, cat. # R-11490) was added to the samples an incubated in darkness during 5 min. Controls and RNA and DNA standards (Ribonuclease A from bovine pancreas, Protease from *Bacillus licheniformis*, Sigma) were included. After the first reading (sensitivity 55, RNA and DNA together), the RNase working solution was added and incubated in darkness during 20 min and then the second reading were taken. RNase solution was found to increase the background fluorescence somewhat and this increase was factored into the calculations (Gorokhova and Kyle, 2002).

2.5.2. Fitness analyses

Survivorship (L_x) and mortality rate (q_x) were computed for all replicates using life-table methods (Daniels and Allan, 1981). Survival percentage after 8 (*E. rapax*) or 7 days (*C. carinata*) of exposure were used to calculate the toxic parameter LpH_{50}, which was defined as the pH that caused a lethal effect in 50% of the population. This parameter was calculated by the linear interpolation method for lethal toxicity (Basallote et al., 2014). Longevity was expressed as the day at which 50% of the original population, exposed to different pH treatment survived and was also calculated by the linear interpolation method. Fertility was considered as the number of new-born individuals per female.

2.6. Statistical analyses

The lack of significant variations along time within pH treatment was checked by a one-way analysis of variance (ANOVA) for the *C. carinata* experiment and a Kruskal-Wallis test for the *E. rapax* experiment after cheeking the results for normality (Kolomogorov–Smirnov test).

Statistical comparisons of mortality rate and life expectancy in *E. rapax* and *C. carinata* were made between the pH groups and at different time points using a two-way ANOVA after checking homogeneity of variances (Levene test) and normality (Kolmogorov–Smirnov test).

Table 1
Physico-chemical parameters of seawater for the different treatments on both experiments. Values are means ± SD for pH (NBS scale), salinity, oxygen, temperature, total alkalinity (TA), carbon dioxide partial pressure (pCO_2), bicarbonate and carbonate ion concentration ($[HCO^{3-}]$) and ($[CO_3^{2-}]$), and calcite and aragonite saturation states (Ω_{cal} and Ω_{ara}).

Parameter	*Cyathura carinata*			*Elasmopus rapax*		
Temperature (°C)		19.5 ± 0.8			20.4 ± 0.38	
Salinity		34.8 ± 0.56			34.3 ± 0.99	
DO		> 80%			> 92%	
pH treatment	Control	7	6.5	Control	7.5	7
pH_{NBS}	7.98 ± 0.09	6.97 ± 0.17	6.65 ± 0.23	7.94 ± 0.08	7.54 ± 0.22	6.95 ± 0.15
TA (μmol/kg)	3000	4590	7357	2764	3048	4191
HCO^{3-} (μmol/kg)	2608	4477	7300	2469	3547	4136
CO_3^{2-} (μmol/kg)	169.27	51.28	26.9	120.63	75.8	23.93
pCO_2 (μatm)	1274	12,386	62,855	423	1550	5984
Ω_{Cal}	4.28	1.30	0.68	2.90	1.90	0.57
Ω_{Ara}	2.85	0.86	0.45	1.82	0.78	0.36

For non-homogeneous variances, an ANOVA was used after setting the p-value equal to 0.01 to reduce the chance of a type-I error (Underwood, 1997). Post-hoc comparisons were established through a Student–Newman–Keuls test (SNK). The RNA:DNA ratio comparisons were made by a one-way ANOVA at different pH levels in the *E. rapax* experiment and by a two-way ANOVA between the pH treatments at different time points in the *C. carinata* experiment to compare the length of the surviving crustaceans (*E. rapax* and *C. carinata*) at the end of the experiments following exposures to different pH levels one-way ANOVA was used.

Survival probabilities were estimated using the Kaplan–Meier estimator. A subsequent log–rank test allowed for differences in survival to be detected in different pH exposure groups.

Possible correlations between the RNA:DNA ratio, pCO_2, time and weight of the crustaceans were obtained through Spearman's rank order correlation test. Statistical analyses were performed using SPSS/PC+ statistical packages® (version 15.0).

3. Results

3.1. Physico-chemical parameters

When CO_2 was released from stable geological formations, the seawater chemistry became modified, as reported in Table 1. As expected under low pH conditions, the highest pCO_2 concentrations that were recorded were due to the high concentrations of injected CO_2.

The pH range was between 7.94 and 6.95 in the *E. rapax* experiment and between 7.98 and 6.65 in the *C. carinata* experiment. In general, the seawater pH levels in the different tests chambers were maintained around the target pH, and no differences among pH values were detected throughout the experimental period (for the *E. rapax* experiment, H control = 6.37, H pH 7.5 = 2.37, H pH 7.0 = 2.42, $p > 0.05$; for the *C. carinata* experiment, the F control = 3; F pH 7.0 = 1.43, F pH 6.5 = 2.61, $p > 0.05$).

The calculated ρCO_2 value was 1274 µatm at the highest pH and 62,855 µatm at the lowest pH in the *C. carinata* experiment. The ρCO_2 value calculated in the *E. rapax* experiment was 423 µatm at the highest pH and 5984 µatm at the lowest pH. Bicarbonate and carbonate ion concentrations and pCO_2 at a nominal pH of 7.0 were similar in both experiments, although slightly higher in the *C. carinata* experiment due to the different temperature (nearly 1 °C lower).

3.2. Elasmopus rapax responses

Acidification with CO_2 negatively affected the survival of *E. rapax* (Fig. 1a and Table 2). The lowest survival probabilities were detected in the most acidified treatment, whereas the highest probability occurred in the control group (Fig. 1a).

The crustacean mortality values were used to calculate the toxic parameter (LpH_{50}). An LpH_{50} value of 7.27 ± 0.11 was recorded after 8 days of exposure. The control group exhibited the highest mortality rate on the eighth day of culture ($q_x = 0.3$). On subsequent days for the remainder of the experiment, the rate was low and almost constant. In contrast, the experimental group that was exposed to a pH of 7.0 showed a high mortality rate during the first eight days of culture, reducing their survivorship to 18.2% compare to the control. The experimental group that was exposed to a pH of 7.5 had a high mortality after 15 days of culture, which caused a decline in their survivorship (5.5% compared to the controls).

The average life span of the control individuals was > 22 days. Individuals exposed to a pH of 7.5 were able to survive up to 15 days of culture, while those exposed to a pH of 7.0 could only survive up to 8 days.

Longevity was also found to be significantly reduced by seawater acidification ($F = 153.8$; $p < 0.0001$). Longevity for *E. rapax* in the control group was more than the 22-day experiment. The experimental group that was exposed to a pH of 7.5 showed a reduced longevity of 4.1 days. According to the results from the SNK test ($p < 0.05$), the three experimental groups (control, pH 7.5 and pH 7.0) were significantly different.

The length of the surviving *E. rapax* was also different between the control and pH 7.5 groups ($F = 148.16$, $p < 0.0001$) (Fig. 1b). The individuals in the control group had a maximum length of 9.1 mm, while the average length was 8.51 ± 0.5 mm (1.7 times the initial length) after 22 days of culture. Individuals exposed to a pH of 7.5 reached a maximum length of 6.42 mm, with an average length of 6.03 ± 0.39 mm (1.2 times the initial length) on the same date.

Fertility was also affected by pCO_2. In the control group, the first neonates appeared after 8 days of culture, and a total of 35 neonates were counted at the end the experiment. The individuals exposed to a pH of 7.5 and 7.0 were unable to reproduce, although they could survive until 18 and 13 days, respectively, a period of time longer than necessary for the first reproduction to occur in the control populations.

After 22 days, the RNA:DNA ratio of *E. rapax* in the control group was not significantly different to those exposed to a pH of 7.5 ($F = 2.87$; $p = 0.09$). For the group exposed to a pH of 7.0, a comparison was made after eight days of exposure because the animals in this group had all died by the end of the experiment; after eight days, the RNA:DNA ratios (2.3 ± 0.2) was similar between the control group and the group exposed to a pH of 7.0 (Fig. 1c).

3.3. Cyathura carinata responses

An LpH_{50} value of 6.49 ± 0.09 was recorded for *C. carinata* after 7 days of exposure. Therefore, *C. carinata* was more resistant to acidification than *E. rapax*; its survival was affected at a pH of 6.5, but not at 7.0 (Fig. 1a, and Table 3). Nevertheless, even though the differences between control and pH 7.0 were not statistically significant, the survival was lower in this acidified culture than in the control.

The control and pH 7.0 groups exhibited similar mortality rates during the experimentation period, although isopods exposed to a pH of 7.0 had a higher mortality rate on the seventh day of culture ($q_x = 0.24$). In contrast, those individuals exposed to a pH of 6.5 demonstrated a higher mortality rate compared to the control and the pH 7.0 groups throughout the experiment, with the highest mortality rate at the beginning of the experiment ($q_x = 0.5$).

The average life span of the control isopods and those exposed to acidification was higher than 35 days. However, the number of surviving isopods exposed to a pH of 7.0 decreased by 11.3% compared to the control, and the number of surviving isopods exposed at a pH of 6.5 decreased by 62.4%. Longevity was also significantly reduced by seawater acidification ($F = 17.35$; $p = 0.003$). The average longevity for *C. carinata* in the control group was 33.7 days of culture. Individuals exposed to a pH of 7.0 showed a decrease in longevity to 30.4 days, and isopods exposed to a pH of 6.5 showed a decrease in longevity to 23.3 days. According to the results of the SNK test ($p < 0.05$), the individuals exposed in the control and pH 7.0 experimental groups constituted a homogeneous group that was significantly different from isopods exposed at a pH of 6.5.

The body length of the surviving *C. carinata* after 35 days of experimentation (28 days of exposure plus 7 days of recovery) was different between the control group and two experimental groups ($F = 7.9$; $p = 0.001$) (Fig. 1b). The individuals in the control group had a maximum length of 150 mm (1.57 times the initial length) and an average length of 108.3 ± 21 mm, while those exposed to a pH of 7.0 reached a maximum length of 113 mm (1.2 times the initial length) with an average of 85.8 ± 17.4 mm. The isopods cultured at a pH of 6.5 had a maximum length of 100 mm (1.05 times the initial length), with an average length of 79.2 ± 9.3 mm. According to the results of the SNK test ($p < 0.05$), controls were significantly different from both pH experimental groups.

The fertility of *C. carinata* was also affected by pCO_2. Although the

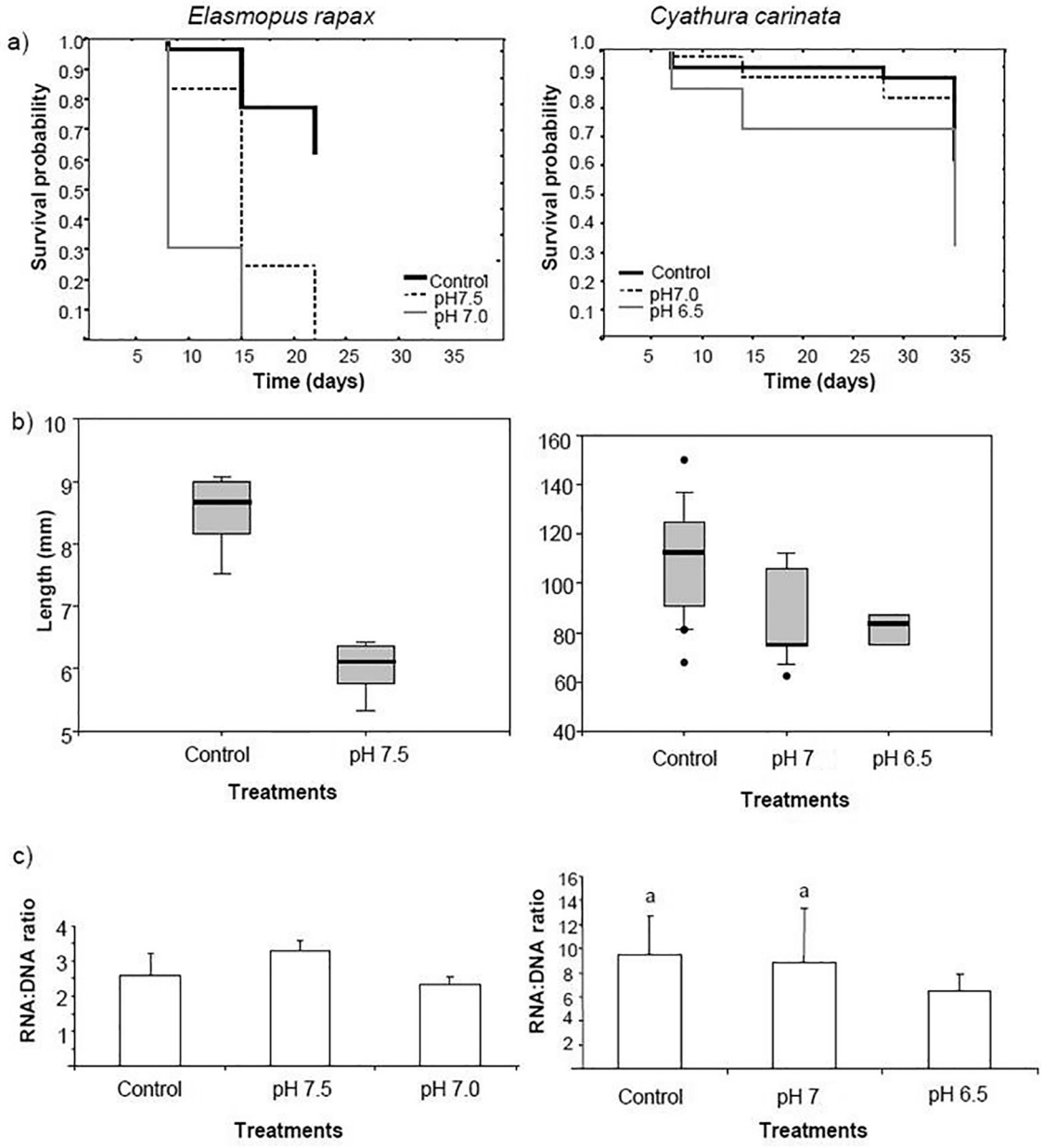

Fig. 1. a) Survival probabilities of both species projected with the Kaplan–Meier estimator. b) Box and whisker plots show the length (mm) of both species at different pH after 22 and 35 days of experiment, respectively. c) RNA/DNA ratios mean value (± SD) obtained in both species (*E. rapax*: control, pH 7.5 (22 days of exposure) and pH 7.0 (8 days of exposure); *C. carinata*: control, pH 7.0 and pH 6.5 after 28 days of exposure). Lowercase denotes homogeneous groups according to SNK test ($p < 0.05$).

Table 2
Log–rank test comparing survival probabilities of *E. rapax* in the different treatments. Survival probabilities were estimated with the Kaplan–Meier estimator.

Source	χ^2	*p*
Control × pH 7.5	32.82	< 0.0001
Control × pH 7	77.49	< 0.0001
pH 7.5 × pH 7	29.55	< 0.0001

Table 3
Log–rank test comparing survival probabilities of *C. carinata* in the different treatments. Survival probabilities were estimated with the Kaplan–Meier estimator.

Source	χ^2	P
Control × pH 7	0.33	0.5647
Control × pH 6.5	16.66	< 0.0001
pH 7.0 × pH 6.5	12.70	0.0004

first ovigerous females appeared in the control and pH 7.0 groups at the same time (on the seventh day of culture), the eggs of those ovigerous females exposed to a pH of 7.0 were not observed on the following day. No ovigerous females were observed in the group exposed to a pH of 6.5.

A significant difference was observed in the RNA:DNA ratio between the two experimental groups ($F = 6.54$; $p = 0.003$) and exposition time ($F = 11.42$, $p < 0.0001$). There was a significant interaction between the pH level and exposure time ($F = 6.39$, $p < 0.0001$). In fact, each of the exposure groups behaved differently as time progressed. According

to the results of the SNK test ($p < 0.05$), the RNA:DNA ratio decreased after 14 days of exposure to a pH of 7.5, and then remained constant throughout the duration of the experiment being its value very similar to that achieved by the control populations. In the group exposed to a pH of 7.0, the RNA:DNA ratio were variable throughout the experiment, while in group exposed to a pH of 6.5, there were no significant differences.

There was a negative correlation between pCO_2 and RNA:DNA ratio measured in the *C. carinata* through the period of exposure ($p = 0.01$; $r = -0.43$), which indicates an acidification–response correlation; however, no other correlations were found in this study.

4. Discussion

Potential CO_2-related acidification scenarios such as the leaks related to the CCS could create more rapid and stronger acidification events than those induced by exchanges between the atmosphere and the ocean surface (Díaz-García et al., 2017). Shallow CO_2 injection experiments under controlled conditions have been previously conducted at pH values consistent with the lowest pH values used in our experiments (Denchik et al., 2014; Jones et al., 2014; Shitashima et al., 2015). Increasing alkalinity has also been identified at the start time of the injection (Shitashima et al., 2015); although the highest pCO_2 value was around 1200 µatm, which differs from the highest pCO_2 values presented in the present paper. These pCO_2 values are in accordance with those obtained in previous experiments that used the same pH values and CO_2 injection system (i.e., Basallote et al., 2015, 2017; Díaz-García et al., 2016;Rodríguez-Romero et al., 2014). A reason for the differences in the pCO_2 values might lie in controlled laboratory conditions ("closed system") (Díaz-García et al., 2016) which did not contemplate the CO_2 plume migration paths and the tidal periodicity. That could have an influence on the pH fluctuations in 'in situ' CO_2-related acidification scenarios (Shitashima et al., 2008). In spite of the possible buffering capacity of the sediment used in the *C. carinata* experiment, similar patterns of increasing pCO_2 were found in both experiments, with the highest pCO_2 recorded at the lowest values of pH. Although the buffering capacity of sediments can limit the pH variations in the ecosystem, in noncarbonated sediments or for large releases of CO_2, the buffering capacity of the sediments might be exceeded (Lichtschlag et al., 2015). A similar pattern of CO_3^{2-} reduction with a decreasing pH and similar saturation states of aragonite (Ω_{Arag}) and calcite (Ω_{Cal}) were observed in the two experiments (Table 1). A calcium carbonate saturation state lower than one is considered especially harmful for calcifying organism because this water becomes under-saturated in carbonate minerals, which is vital for their skeleton and shell building (Fabry et al., 2008). Conversely, crustaceans are generally more tolerant to acidification, and the process of calcification seems not to be negatively affected in some species (Hauton et al., 2009; Kurihara et al., 2008; Munguia and Alenius, 2013; Spicer et al., 2007). Nevertheless, maintaining the calcification rates could imply a high-energy cost for the organisms (Hendriks et al., 2015; Long et al., 2013).

As carbon dioxide is a toxic substance at elevated concentrations (Hsieh et al., 2005), there is a pH threshold (or pCO_2) that marine organisms can support (Basallote et al., 2014). If there is a pH reduction below this value, then lethal effects on organisms appear. This information allows us to quantify rapid CO_2-related acidification and it could positively contribute to environmental risk assessment studies like in CCS operations. The pCO_2 threshold that the estuarine isopod *C. carinata* can support is 6.49 ± 0.09. The marine amphipod *E. rapax* was more sensitive and was only able to support pH threshold of 7.27 ± 0.11. The differences in the pCO_2 thresholds may be due to the different life strategies of the two organisms because sediment-dwelling fauna are less vulnerable to lower pH conditions than those who live in the water column or on the sediment surface (Widdicome et al., 2011). This suggestion is supported by the lack of acute effects in the sediment-dwelling polychaetes *Hediste diversicolor* (Rodríguez-Romero et al., 2014) or *Nereis virens* (Batten and Bamber, 1996; Widdicombe and Needham, 2007), exposed to pH levels above 6.5 over the short or medium term, compared to the high mortality rate found in the crab *Carcinus maenas* when exposed to pH 6.15 during 10 days (Rodríguez-Romero et al., 2016). In contrast, the survival of the amphipod *Corophium volutator* was not affected when incubated in seawater with a pH of up to 0.5 units (Hauton et al., 2009). Acute and chronic lethal effects on nauplii and adult benthic copepods (*Tigriopus japonicus* or *Tigriopus* sp.) caused by elevated CO_2 occur at pH values ranging from 5.70 to 5.85 (Kita et al., 2013) and Lee et al., 2016).

In the present study, reduced survival was observed at the lowest pH exposures (that is, a reduction in the average lifespan of 18.2% at pH 7.0 for *E. rapax* and of 62.4% at pH 6.5 for *C. carinata* compared to the control). This provides evidence that a reduction of the pH in seawater ecosystems can have dramatic consequences on crustacean populations, and possibly on other organisms. A similar decrease in longevity with decreasing pH occurred in both species. *E. rapax* was particularly vulnerable—all amphipods died within 15 days at a pH of 7.0. In *C. carinata* the effect was not significant until the pH was reduced to 6.5.

The above findings mirror the different patterns of increased mortality observed when these marine crustaceans are exposed to water with a low pH. The increase in mortality was evident almost immediately (approximately 10 days) for *E. rapax* at both tested pHs, but without any notable difference in mortality between the control group and the group exposed to a pH of 7.0 in the *C. carinata* experiment. Such delay in the response has also been observed in shrimps (such as *Palaemon pacificus, P. elegans* and *P. serratus*) and crabs (such as *Necora puber, Cancer magister* and *Paralithodes platypus*), which may have the ability to compensate for hypercapnia (excess CO_2 in blood) for a few days (Dissanayake et al., 2010; Kurihara et al., 2008; Long et al., 2017; Pane and Barry, 2007; Spicer et al., 2007). However, that compensation may come at an energetic cost that eventually results in less growth or higher mortality rates when energy reserves are depleted. The average length of surviving organisms in both *E. rapax* and *C. carinata* decreased significantly at all pH levels due to the higher mortality of the large and more mature individuals, to the decreased growth rate following pH reduction or to both. Reduction in growth when pH decreases has been observed in some crustaceans (Arnold et al., 2009; Fitzer et al., 2012; Kurihara et al., 2008; Long et al., 2013) but not in others (Almén et al., 2017; Findlay et al., 2010; Hauton et al., 2009).

Although the RNA:DNA ratio, a common metabolic indicator for crustaceans (Bullejos et al., 2014; Speekmann et al., 2007; Vrede et al., 2002), was reduced for both species, which suggests that rates of transcription and growth were compromised, no significant differences were found between control populations and those exposed to lower pH. Similarly, Parra et al. (2016) did not found significant differences in the RNA:DNA ratio when the cladoceran *Daphnia magna* was exposed to different pH levels. Franke and Clemmesen (2011) found a significant negative linear relationship between pCO_2 and the RNA:DNA ratio in the Atlantic herring *Clupea harengus*, but this correlation was no longer detected when the highest exposure group was excluded ($pCO_2 = 4635$ µatm) from the statistical analysis. Gobler and Talmage (2013) demonstrated a negative correlation between RNA:DNA ratio and shell-based growth rates of two mussels species at 250 µatm and 1500 µatm of CO_2. Furthermore, Büscher et al. (2017), studying the interaction between warming and acidified waters on the coral *Lophelia perthusa* under different food availabilities, showed diverging responses of the RNA:DNA ratio to the different treatment conditions. No consistent pCO_2 or temperature effects were observed in relation to the RNA:DNA, but a statistically significant interaction between all three factors (temperature, CO_2, and food) was identified. The low correlation between pCO_2 and the RNA:DNA ratio measured in *C. carinata* through the exposure duration of this study might indicate that other factors can influence the result and interpretation of the RNA:DNA ratio in this isopod under acidified water conditions. Some previous

literature has reported complex interactions between temperature and other environmental variables that may affect nucleic acid content and that may act as additional homogenising factors (Lagos et al., 2015), such as gender (Chícharo and Chícharo, 2008), tissue type and size classes (Gobler and Talmage, 2013).

The reproduction of both crustacean species in the present study was strongly impaired—neither species was able to reproduce successfully under acidified conditions. Reproduction does not occur without costs, both in terms of post-reproductive survival and future reproductive potential (Sterns, 1989). Carrying brood is likely to be energetically costly. If the energy status of a female was reduced (e.g., by stress) to the extent that by incubating a brood she jeopardises her own survival, then her overall fitness may be increased by sacrificing the brood and reproducing at a later date. Regardless of the length of their life cycles, as both crustacean species are iteroparous, ultimately it is not the number of surviving offspring in a single brood that is important but the total number of surviving offspring produced in the lifetime of a female. This is supported by the "disappearance" of the ovigerous *E. rapax* females (probably by absorption of the eggs or the death of ovigerous females) when animals were exposed to a pH of 7.0. Indeed, the effect of zinc in the amphipod *Corophium volutator* was to change the energy from reproduction to the survivorship of females (Conradi and Depledge, 1999). These authors suggested that the broods were aborted due to the condition of the female rather than her offspring. Similarly, Moulin et al. (2011) concluded that fertilisation is one of the most critical phases of the echinoderm *Paracentrotus lividus* affected by pH changes. Parra et al. (2016) demonstrated that neonate production and, consequently, population rates and secondary production were greatly reduced by low pH conditions in *Daphnia magna* (pH 7.0). Nevertheless, exposure to CO_2-acidified seawater (pH 7.50–7.32) slightly slowed development in embryonic amphipods (Hauton et al., 2009) or in the copepod *Arcatia tseuensis* (Kurihara and Ishimatsu, 2008), with no affection in the number of hatchlings produced in both species. Kurihara et al. (2004) reported that a decreasing trend of hatching success with increasing acidity in the closely-related copepod *Arcatia steueri*, with no significant differences when the pH reduced to 7.31. Delays in hatching, spawning and development and immobilisation of benthic copepods occurred at pH values ranging from 6.04 to 6.31 (Kita et al., 2013; Lee et al., 2016). Under chronic elevated CO_2 conditions, metabolic suppression can occur and typically that means to shut down expensive processes (e.g., protein synthesis). The suppression of metabolism reduces growth and reproductive output leading to an effective diminish the survival of the exposed populations on longer time scales (Seibel and Fabry, 2003).

Crustaceans that tolerate ocean acidification will likely still divert energy away from key biological processes—such as immune function or other repair systems, growth and reproduction—toward compensatory processes and ion homeostasis (Hernroth et al., 2011; Munguia and Alenius, 2013; Roberts et al., 2013). Regardless of the underlying processes altered and the energetic pathways modified in the two crustacean species in the present study, it cannot be excluded that acclimatisation to low pH comes at a great cost, based on the observation that neither species could reproduce.

5. Conclusions

The different responses with respect to rapid CO_2-related acidification on the two crustacean species in the present study demonstrate how highly variable this group is in terms of coping with pH changes. Our analyses revealed a strong negative effect of CO_2-related acidification on marine crustaceans, despite the variation in the sensitivity of taxonomic groups. Our analyses also highlighted the important implications of different sensitivities for the mitigation of environmental problems arising from man-made CO_2 emissions, such as CCS technologies. Consistent with previous publications, our results also show some variability in the relationship between the RNA:DNA ratio and pCO_2. However, regardless of the underlying processes and the energetic pathways that could lead to differences between the two crustacean species, the long life span isopod and the short life span amphipod, the results are nearly the same: a failure in reproduction could lead to the local extinction of these species.

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Conflict of interest

The authors declare that they have no conflict of interest.

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